

SBQID1

ALIGNMENTS

RESULT 1  
AAF57290  
ID AAF57290 standard; DNA; 3231 BP.  
XX  
AC AAF57290;  
XX  
DT 29-MAY-2001 (first entry)  
XX  
DE M. bovis Dalton 2d RTX toxin A subunit encoding DNA.  
XX  
KW Moraxella; antigen; immune response; infection; RTX toxin; vaccine;  
KW antibacterial; ds.  
XX  
OS Moraxella bovis.  
XX  
FH Key  
FT CDS Location/Qualifiers  
FT 232..3015  
FT /\*tag= a  
FT CDS /product= "RTX toxin A subunit"  
FT 1..195  
FT /\*tag= b  
FT CDS /note= "partial coding region of RTX toxin C subunit"  
FT 3080..3250  
FT /\*tag= c  
FT /note= "partial coding region of RTX toxin B subunit"  
XX  
PN WO200116172-A1.  
XX  
PD 08-MAR-2001.  
XX  
PF 31-AUG-2000; 2000WO-AU01048.  
XX

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PR	31-AUG-1999:	99AU-0002571.
XX	(CSIR ) COMMONWEALTH SCI & IND RES. ORG.	
PA	(UYME ) UNIV MELBOURNE.	
XX		
PI	Farn J, Strugnelli R, Tennent J;	
XX		
DR	WPI: 2001-235092/24.	
DR	P-PSDB: AAB62110, AAB62112, AAB62113.	
XX		
PT	Novel Moraxella bovis antigen useful in compositions for raising immune response in an animal, has protease, lipase or hemolysin activity	
CC	Claim 32; Fig 5; 60pp; English.	
XX		
PS	The invention relates to new Moraxella bovis antigens and nucleic acid sequences encoding these antigenic polypeptides. The antigenic polypeptides and polynucleotides are useful for raising an immune response in an animal directed against Moraxella, preferably against M. bovis or M. catarrhalis, and for treating Moraxella infections. The present sequence represents the nucleotide sequence of the A subunit of the RTX toxin from M. bovis Dalton 2d.	
CC		
XX		
SO	Sequence 3231 BP; 1072 A; 478 C; 671 G; 1010 T; 0 other;	
Query Match	99.9%; Score 2782.4; DB 22; Length 3231;	
Best Local Similarity	100.0%; Pred. No. 0;	
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DB	232 atgtccaatataaatgttaattcaatcattcaatcaagcagctgttaattcaacaagtct 291	
OY	61 ggattaaaaatcttacttggctctctcccaagaattatgatccgcaaaaagttggact 120	
DB	292 ggattaaaaatcttacttggctctctcccaagaattatgatccgcaaaaagttggact 351	
OY	121 ttaattgtatttatcaagctgtctgaatgaattagatattgtctcgttttagcaaaagact 180	
DB	352 ttaattgtatttatcaagctgtctgaatgaattagatattgtctcgttttagcaaaagact 411	
OY	181 aatcaccttgaaaacagcaaaaatactctgtgacacagtaaatcagttctctctcaca 240	
DB	412 aatcaccttgaaaacagcaaaaatactctgtgacacagtaaatcagttctctctcaca 471	
OY	241 caactgttattgtctattcttgcacaacaattagaagaagttcttcaaaaacattctacc 300	
DB	472 caactgttattgtctattcttgcacaacaattagaagaagttcttcaaaaacattctacc 531	
OY	301 aataagtttagccaaagggttagacagtttagaagaatatatgacgtgaattagtaaga 360	
DB	532 aataagtttagccaaagggttagacagtttagaagaatatatgacgtgaattagtaaga 591	
OY	361 agtaatgtatataccaacttaagcctcttcttggcgactgcaattagcgggtatagaact 420	
DB	592 agtaatgtatataccaacttaagcctcttcttggcgactgcaattagcgggtatagaact 651	
OY	421 gattcttcaatcaaaaaagtgatagtcgcacgcvgatgctttgctctaaagctgtattgac 480	
DB	652 gattcttcaatcaaaaaagtgatagtcgcacgcvgatgctttgctctaaagctgtattgac 711	
OY	481 ttcatttaagagaataattgtgatactactctagagtaaccacaagaatgaacatttct 540	
DB	712 ttcatttaagagaataattgtgatactactctagagtaaccacaagaatgaacatttct 771	
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DB	772 tccacgttagcaaaagttagtgcttactatatacgcgagcttaaaggtcttctataataga 831	
OY	601 aacaaagtgcacaaacttaaatcttctcaaaaacaactctgtgtttggaaataattactgct 660	
DB	831 aacaaagtgcacaaacttaaatcttctcaaaaacaactctgtgtttggaaataattactgct 891	

QY	661	ttgtatcagcgatctcttcgaagccttgcttgtagcgataaanaagatcgagcttgcaaa	720
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QY	721	aaagtctcgaaagttttggaattaaagcaatccaagttatcggtaaatgaacaaagcaat	780
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QY	781	tcctcaatagtctttagacacaacgltgtctgcctgcgtctatccaactctgtgtgtgt	840
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QY	901	gataatccaatctgataatgtgcctctgtagagtttcgcaacaacatccgaaattctgc	960
Db	1132	gataatccaatctgataatgtgcctctgtagagtttcgcaacaacatccgaaattctgc	1191
QY	961	taagaatgggagatcaatctatctgctctgaatalcagcgtgtgtgtacttatgaagcttca	1020
Db	1192	taagaatgggagatcaatctatctgctctgaatalcagcgtgtgtgtacttatgaagcttca	1251
QY	1021	ttaaactcaaatgaataagcgatattagtggaagtttcgcgcgtgtttccgcctctctgta	1080
Db	1252	ttaaactcaaatgaataagcgatattagtggaagtttcgcgcgtgtttccgcctctctgta	1311
QY	1081	ggaactcgtctgtctgtagcagcgatctgacatactagctgtcagcgtgttaacgaattgtact	1140
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Db	1372	ggaattcttagaagcgtcttaaacagcgaaatgtttggaagtggttgataacggtttaaagtt	1431
QY	1201	aaaattcttagagtgtaggaagaacaaatctggtgcgcgacaactatttgaataaagcctat	1260
Db	1432	aaaattcttagagtgtaggaagaacaaatctggtgcgcgacaactatttgaataaagcctat	1491
QY	1261	tcctcgttatgtctgtcttatcttagcctaataacttaaaatttltgtctgagcttaacaagaag	1320
Db	1492	tcctcgttatgtctgtcttatcttagcctaataacttaaaatttltgtctgagcttaacaagaag	1551
QY	1321	ttggaagctgaaagctgttatcttgcaatacccaacaagcttgggataataatatgtgtgag	1380
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QY	1381	ttagaaggttatctacaatatctgggtggaacgcatctaaagcggaaagaactctatactcagatgt	1440
Db	1612	ttagaaggttatctacaatatctgggtggaacgcatctaaagcggaaagaactctatactcagatgt	1671
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on Sep 16 16:43:25 2002

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Qy 1801 aatattgatgggtggagatggacacgatcgtgtcttctatagtaaagacggaggatttgg 1860  
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Qy 1981 cgtactgaaactatccagatcgtgattatgaattaaagaaagttgggtatggttatcag 2040  
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Qy 2041 tctaccgataatttgaatcagtagaagaagtaattggttctcaatttaagtatttc 2100  
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Qy 2281 gatgtctatatctttcgaaaggtgatggtaatgatactttgtacgatggcagggcaat 2340  
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Qy 2341 gataaatttagcatttgcagatgcaaatatctgatattatgattgaacgtaccaaagag 2400  
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Qy 2401 ggtattatagttaaacgaatgatcattcaggtagttatcaacataccaagatggtacata 2460  
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Db 2692 acatcaaatttacaaaattatcaaagtaataaaacagatcataaattgagcaactaatt 2751  
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Qy 2581 gatggtacagtaattacatctcaagaattgaaaaagcttgctgatgagaataagagccaa 2640  
Db 2812 gatggtacagtaattacatctcaagaattgaaaaagcttgctgatgagaataagagccaa 2871  
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Db 2932 tttggtacagcaaatagtgtagttctaacgccttacagccaattacacaaccaactcaa 2991  
Qy 2761 ggaattttggctccaagtgttag 2784  
Db 2992 ggaattttggctccaagtgttag 3015

SEQID  
6

# ALIGNMENTS

RESULT 1  
AAB62110  
ID AAB62110 standard; Protein; 927 AA.  
XX  
AC AAB62110;  
XX  
DT 29-MAY-2001 (first entry)  
XX  
DE M. bovis Dalton 2d RTX toxin A subunit.  
XX  
KW Moraxella; antigen; immune response; infection; RTX toxin; v  
KW antibacterial; A subunit.  
XX  
OS Moraxella bovis.  
XX  
PN WO200116172-A1.  
XX  
PD 08-MAR-2001.  
XX  
PF 31-AUG-2000; 2000WO-AU01048.  
XX  
PR 31-AUG-1999; 99AU-0002571.  
XX  
PA (CSIR ) COMMONWEALTH SCI & IND RES ORG.  
PA (UYME ) UNIV MELBOURNE.  
XX  
PI Farn J, Strugnell R, Tennent J;  
XX  
DR WPI; 2001-235092/24.  
DR N-PSDB; AAF57290.  
XX  
PT Novel Moraxella bovis antigen useful in compositions for raising immune  
PT response in an animal, has protease, lipase or hemolysin activity -  
XX



88

PS Claim 26; Fig 5; 60pp; English.  
XX  
CC The invention relates to new Moraxella bovis antigens and nucleic acid  
CC sequences encoding these antigenic polypeptides. The antigenic  
CC polypeptides and polynucleotides are useful for raising an immune  
CC response in an animal directed against Moraxella, preferably against  
CC M. bovis or M. catarrhalis, and for treating Moraxella infections. The  
CC present sequence represents the amino acid sequence of the A subunit of  
CC the RTX toxin from M. bovis Dalton 2d.  
XX  
SQ Sequence 927 AA;

Query Match 100.0%; Score 57; DB 22; Length 927;  
Best Local Similarity 100.0%; Pred. No. 0.2;  
Matches 12; Conservative 0; Mismatches 0; Indels 0; Gaps 0;

Qy 1 FLSELNKELEAE 12  
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Db 433 flselnkeleae 444

58613

RESULT 1  
 AAB62110  
 ID AAB62110 standard; Protein: 927 AA.  
 XX  
 AC AAB62110;  
 XX  
 DT 29-MAY-2001 (first entry)  
 XX  
 DE M. bovis Dalton 2d RTX toxin A subunit.  
 XX  
 KW Moraxella; antigen; immune response; infection; RTX toxin; vaccine;  
 KW antibacterial; A subunit.  
 XX  
 OS Moraxella bovis.  
 XX  
 PN WO200116172-A1.  
 XX  
 PD 08-MAR-2001.  
 XX  
 PF 31-AUG-2000; 2000WO-AU01048.  
 XX  
 PR 31-AUG-1999; 99AU-0002571.  
 XX  
 PA (CSIR ) COMMONWEALTH SCI & IND RES ORG.  
 PA (UYME ) UNIV MELBOURNE.  
 XX  
 PI Farn J, Strugnell R, Tennent J;  
 XX  
 DR WPI; 2001-235092/24.  
 DR N-PSDB; AAF57290.  
 XX  
 PT Novel Moraxella bovis antigen useful in compositions for raising immune  
 PT response in an animal, has protease, lipase or hemolysin activity  
 XX  
 PS Claim 26; Fig 5; 60pp; English.  
 XX  
 CC The invention relates to new Moraxella bovis antigens and nucleic acid  
 CC sequences encoding these antigenic polypeptides. The antigenic  
 CC polypeptides and polynucleotides are useful for raising an immune  
 CC response in an animal directed against Moraxella, preferably against  
 CC M. bovis or M. catarrhalis, and for treating Moraxella infections. The  
 CC present sequence represents the amino acid sequence of the A subunit of  
 CC the RTX toxin from M. bovis Dalton 2d.  
 XX  
 SQ Sequence 927 AA;  
  
 Query Match 100.0%; Score 77; DB 22; Length 927;  
 Best Local Similarity 100.0%; Pred. No. 7.4e-05;  
 Matches 14; Conservative 0; Mismatches 0; Indels 0; Gaps 0;  
  
 Qy 1 FNDIFHSGEGDDL 14  
 Db 705 fndifhsgegddl 718

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DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,  
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,  
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,  
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,  
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- (30) Priority Data:  
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**STRUGNELL, Richard** [AU/AU]; 17 Salisbury Grove,
- (84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
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IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,  
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:  
— With international search report.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



**WO 01/16172 A1**

(54) Title: **VACCINE ANTIGENS OF MORAXELLA**

(57) Abstract: The present invention relates to antigens of *Moraxella*, in particular, *Moraxella bovis*, nucleic acid sequences encoding these antigens and formulations for use in raising an immune response against *Moraxella*.

## ***Vaccine antigens of Moraxella***

### **FIELD OF THE INVENTION**

The present invention relates to antigens of *Moraxella*, in particular, *Moraxella bovis*, nucleic acid sequences encoding these antigens and  
5 formulations for use in raising an immune response against *Moraxella*.

### **BACKGROUND OF THE INVENTION**

Infectious bovine keratoconjunctivitis (IBK) is an economically important disease of cattle caused by the Gram-negative coccobacillus  
10 *Moraxella bovis*. More commonly known as pinkeye, IBK is a highly contagious ocular infection which may range from mild conjunctivitis to severe ulceration, corneal perforation and blindness. Therapeutic and preventative measures have limited success in controlling IBK and a vaccine which will prevent the disease is required. A number of factors contribute to  
15 the virulence of the organism, the two most important attributes so far identified are the expression of pili, and the ability to produce haemolysin. Seven different serogroups of *M. bovis* strains isolated in Australia, Great Britain and the USA have been characterised, based on pilus types (1). An efficacious pilus-based vaccine must contain a sufficient quantity of pili from  
20 all seven serotypes to be fully protective, because of a lack of cross protection between serotypes (2, 3). Expression of all seven pilus serotypes at levels high enough to be useful for commercial vaccine preparation has not been achieved.

The ideal vaccine candidate to stimulate protection against *M. bovis*  
25 would be a molecule that is highly-conserved among all strains of this species. Possible candidates are haemolysin, protease, lipase and/or phospholipase (4) enzymes produced by *M. bovis*. For example, a partially purified cell-free supernatant from one haemolytic strain of *M. bovis* has been shown to confer significant protection against heterologous, wild-type  
30 challenge (5). The possibility that a haemolysin could be conserved across all seven serotypes of *M. bovis* makes it a potential vaccine candidate against IBK. However, researchers have so far been unable to either clone the gene encoding the haemolysin or purify the protein to homogeneity. Nevertheless, any or all of these molecules, alone or in combination, could prove useful for  
35 the generation of an effective vaccine against IBK.

**SUMMARY OF THE INVENTION**

In a first aspect the present invention consists in a polypeptide, the polypeptide having an amino acid sequence as set out in SEQ. ID. NO. 1 from amino acid 37 to 1114, or a sequence having at least 50% identity thereto, or a functional fragment thereof.

In a preferred embodiment the polypeptide has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 1.

In a further preferred embodiment of the first aspect of the present invention the polypeptide has protease activity.

In a second aspect the present invention consists in a nucleic acid molecule, the nucleic acid molecule encoding the polypeptide of the first aspect of the present invention.

In a third aspect the present invention consists in a nucleic acid molecule comprising a sequence as set out in SEQ. ID. NO. 2 or a sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.

In a preferred embodiment the nucleic acid molecule has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 2.

In a fourth aspect the present invention consists in a composition for use in raising an immune response in an animal, the composition comprising the polypeptide of the first aspect of the present invention or the nucleic acid sequence of the second aspect of the present invention and optionally a carrier and/or adjuvant.

In a fifth aspect the present invention consists in a polypeptide, the polypeptide having an amino acid sequence as set out in SEQ. ID. NO. 3 from amino acid 26 to 616, or a sequence having at least 50% identity thereto, or a functional fragment thereof.

In a preferred embodiment the polypeptide has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 3 from amino acid 26 to 616.

In a further preferred embodiment of the fifth aspect the polypeptide has lipase activity.



In a sixth aspect the present invention consists in a nucleic acid molecule, the nucleic acid molecule encoding the polypeptide of the fifth aspect of the present invention.

5 In a seventh aspect the present invention consists in a nucleic acid molecule comprising a sequence as set out in SEQ. ID. NO. 4 or a sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.

10 In a preferred embodiment the nucleic acid molecule has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 4.

15 In an eighth aspect the present invention consists in a composition for use in raising an immune response in an animal, the composition comprising the polypeptide of the fifth aspect of the present invention or the nucleic acid sequence of the sixth aspect of the present invention and optionally a carrier and/or adjuvant.

In a ninth aspect the present invention consists in a polypeptide, the polypeptide having an amino acid sequence as set out in SEQ. ID. NO. 5, or a sequence having at least 60% identity thereto, or a functional fragment thereof.

20 In a preferred embodiment the polypeptide has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 5.

In a further preferred embodiment of the ninth aspect the polypeptide has haemolysin activity.

25 In a tenth aspect the present invention consists in a nucleic acid molecule, the nucleic acid molecule encoding the polypeptide of the ninth aspect of the present invention.

30 In an eleventh aspect the present invention consists in a nucleic acid molecule comprising a sequence as set out in SEQ. ID. NO. 6 or a sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.

In a preferred embodiment the nucleic acid molecule has a sequence of at least 70%, more preferably at least 80% and most preferably at least 90% identity with the sequence shown in SEQ. ID. NO. 6.

35 In a twelfth aspect the present invention consists in a composition for use in raising an immune response in an animal, the composition comprising

the polypeptide of the ninth aspect of the present invention or the nucleic acid sequence of the tenth aspect of the present invention and optionally a carrier and/or adjuvant.

5 The term "functional fragment" as used herein is intended to cover fragments of the polypeptide which retain at least 10% of the biological activity of the complete polypeptide. In particular this term is used to encompass fragments which show immunological cross-reactivity with the entire polypeptide, eg ligands which react with the fragment also react with the complete polypeptide.

10 In a thirteenth aspect the present invention consists in a composition for use in raising an immune response in an animal directed against *Moraxella*, the composition comprising at least one polypeptide selected from the group consisting of the polypeptides of the first, fifth and ninth aspects of the present invention and optionally including an adjuvant or carrier.

15 In a preferred embodiment the composition includes the polypeptide of the ninth aspect of the present invention and either one of, or preferably both, the polypeptides of the first and fifth aspects of the present invention.

In a preferred embodiment the *Moraxella* is *M. bovis* or *M. catarrhalis*, most preferably *M. bovis*.

20 In a fourteenth aspect the present invention consists in an antibody raised against a polypeptide selected from the group consisting of the polypeptides of the first, fifth and ninth aspects.

As will be readily appreciated by the person skilled in this field the polypeptides and antibodies of the present invention and probes derived  
25 from the nucleotide sequences can be used as diagnostic reagents in determining *Moraxella*, in particular, *M. bovis* infection. For example, the polypeptides and antibodies can be used in ELISA based assays whilst the probes can be used in PCR based assays. The probes will be of a length to provide the required level of specificity and will typically be at least 18  
30 nucleotides in length.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements,  
35 integers or steps.

**BRIEF DESCRIPTION OF THE FIGURES**

Figure 1: Nucleotide and amino acid sequence of a protease from *M. bovis* Dalton 2d. A putative promoter sequence is singly underlined. A putative ribosome binding site is shown in bold and underlined. A putative start codon is shown in bold. Putative transcription terminator sequences are indicated by inverted arrows. Numbering for both the nucleotide and amino acid sequences are shown on the left hand side.

Figure 2: Nucleotide and amino acid sequence of a lipase from *M. bovis* Dalton 2d. A putative promoter sequence is singly underlined. A putative ribosome binding site is shown in bold and underlined. A putative start codon is shown in bold. Putative transcription terminator sequences are indicated by inverted arrows. Numbering for both the nucleotide and amino acid sequences are shown on the left hand side.

Figure 3: Heat stability of the lipase from *M.bovis* when expressed in its recombinant form (pMB1/MC1061). (Heating carried out at 90°C).

Figure 4: Comparison of growth rate and expression levels of the lipase of *M.bovis* when in its (i) native form and (ii) recombinant form. The growth rate is shown as solid bars and the lipase expression levels as open diamonds.

Figure 5: Nucleotide and amino acid sequence of the A subunit of the RTX toxin from *M. bovis* Dalton 2d. A putative ribosome binding site is shown in bold and underlined. A putative start codon is shown in bold. Upstream of the A subunit open reading frame is a portion of the coding region for the C subunit (nucleotide 1 to 195) (corresponding amino acid sequence shown in SEQ ID NO:8) and downstream of the A subunit is a small portion of the B subunit coding region (nucleotide 3080 to 3250) (corresponding amino acid sequence shown in SEQ ID NO:9) . Numbering for both the nucleotide and amino acid sequences are shown on the left hand side.

## DETAILED DESCRIPTION OF THE INVENTION

5 In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following non-limiting Examples.

### General Molecular Biology

10 Unless otherwise indicated, the recombinant DNA techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. 15 Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (Editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until 20 present) and are incorporated herein by reference.

### Protein Variants

25 Amino acid sequence variants can be prepared by introducing appropriate nucleotide changes into DNA, or by *in vitro* synthesis of the desired polypeptide. Such variants include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final protein product possesses the desired characteristics. The amino acid changes also may alter 30 post-translational processes such as altering the membrane anchoring characteristics, altering the intra-cellular location by inserting, deleting or otherwise affecting the transmembrane sequences of the native protein, or modifying its susceptibility to proteolytic cleavage.

35 In designing amino acid sequence variants, the location of the mutation site and the nature of the mutation will depend on characteristic(s) to be modified. The sites for mutation can be modified individually or in

series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of other ligands adjacent to the located site.

5       A useful method for identification of residues or regions for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (*Science* (1989) **244**: 1081-1085). Here, a residue or group of target residues are identified (e.g., charged residues such as Arg. Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged  
10 amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants. Thus, while the site for introducing an amino acid sequence variation is  
15 predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimise the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed variants are screened for the optimal combination of desired activity.

20       There are two principal variables in the construction of amino acid sequence variants; the location of the mutation site and the nature of the mutation. These may represent naturally occurring alleles or predetermined mutant forms made by mutating the DNA either to arrive at an allele or a variant not found in nature. In general, the location and nature of the  
25 mutation chosen will depend upon the characteristic to be modified.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues and typically about 1 to 5 contiguous residues.

Amino acid sequence insertions include amino and/or  
30 carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Other insertional variants include the fusion of the N- or C-terminus of the proteins to an immunogenic polypeptide e.g. bacterial polypeptides such as betalactamase or an enzyme  
35 encoded by the *E. coli trp* locus, or yeast protein, bovine serum albumin, and chemotactic polypeptides. C-terminal fusions with proteins having a

long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, are included.

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the protein molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s). Other sites of interest are those in which particular residues obtained from various species are identical. These positions may be important for biological activity. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

**TABLE 1**

<b>Original Residue</b>	<b>Exemplary Substitutions</b>	<b>Preferred Substitutions</b>
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe norleucine	leu
Leu (L)	norleucine, ile; val; met; ala; phe	ile

Original Residue	Exemplary Substitutions	Preferred Substitutions
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile;	leu
Phe (F)	leu; val; ile; ala	leu
Pro (P)	gly	gly
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

### Mutants, Variants and Homology - Proteins

5 Mutant polypeptides will possess one or more mutations which are deletions, insertions, or substitutions of amino acid residues. Mutants can be either naturally occurring (that is to say, purified or isolated from a natural source) or synthetic (for example, by performing site-directed mutagenesis on the encoding DNA). It is thus apparent that polypeptides of the invention can be either naturally occurring or recombinant (that is to say prepared using recombinant DNA techniques).

10 An allelic variant will be a variant that is naturally occurring within an individual organism.

Protein sequences are homologous if they are related by divergence from a common ancestor. Consequently, a species homologue of the protein will be the equivalent protein which occurs naturally in another species.

15 Within any one species a homologue may exist as numerous allelic variants, and these will be considered homologues of the protein. Allelic variants and species homologues can be obtained by following standard techniques known to those skilled in the art. Preferred species homologues include those obtained from representatives of the same Phylum, more preferably the same

20 Class and even more preferably the same Order.

A protein at least 50% identical, as determined by methods well known to those skilled in the art (for example, the method described by Smith, T.F. and Waterman, M.S. (1981) *Ad. Appl Math.*, 2: 482-489, or Needleman, S.B. and Wunsch, C.D. (1970) *J. Mol. Biol.*, 48: 443-453), to that of the present invention are included in the invention, as are proteins at least 70% or 80% and more preferably at least 90% identical to the protein of the present invention. This will generally be over a region of at least 20, preferably at least 30, contiguous amino acids.

#### 10 **Mutants, Variants and Homology - Nucleic Acids**

Mutant polynucleotides will possess one or more mutations which are deletions, insertions, or substitutions of nucleotide residues. Mutants can be either naturally occurring (that is to say, isolated from a natural source) or synthetic (for example, by performing site-directed mutagenesis on the DNA). It is thus apparent that polynucleotides of the invention can be either naturally occurring or recombinant (that is to say prepared using recombinant DNA techniques).

An allelic variant will be a variant that is naturally occurring within an individual organism.

20 Nucleotide sequences are homologous if they are related by divergence from a common ancestor. Consequently, a species homologue of the polynucleotide will be the equivalent polynucleotide which occurs naturally in another species. Within any one species a homologue may exist as numerous allelic variants, and these will be considered homologues of the polynucleotide. Allelic variants and species homologues can be obtained by following standard techniques known to those skilled in the art. Preferred species homologues include those obtained from representatives of the same Phylum, more preferably the same Class and even more preferably the same Order.

30 A polynucleotide at least 70% identical, as determined by methods well known to those skilled in the art (for example, the method described by Smith, T.F. and Waterman, M.S. (1981) *Ad. Appl Math.*, 2: 482-489, or Needleman, S.B. and Wunsch, C.D. (1970) *J. Mol. Biol.*, 48: 443-453), to that of the present invention are included in the invention, as are proteins at least 80% or 90% and more preferably at least 95% identical to the



polynucleotide of the present invention. This will generally be over a region of at least 60, preferably at least 90, contiguous nucleotide residues.

### Antibody Production

5       The term "antibody" should be construed as covering any specific binding substance having a binding domain with the required specificity. Thus, the term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide including an immunoglobulin binding domain, whether natural or synthetic. Chimaeric  
10       molecules including an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included.

Antibodies, either polyclonal or monoclonal, which are specific for a protein of the present invention can be produced by a person skilled in the art using standard techniques such as, but not limited to, those described by  
15       Harlow et al. *Antibodies: A Laboratory Manual*, Cold Springs Harbor Laboratory Press (1988), and D. Catty (editor), *Antibodies: A Practical Approach*, IRL Press (1988).

Various procedures known in the art may be used for the production of polyclonal antibodies to epitopes of a protein. For the production of  
20       polyclonal antibodies, a number of host animals are acceptable for the generation of antibodies by immunization with one or more injections of a polypeptide preparation, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response in the host animal, depending on the host species, including but not limited to  
25       Freund's (complete and incomplete), mineral gels such as aluminium hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

30       A monoclonal antibody to an epitope of a protein may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256, 493-497), and the more recent human B-cell  
35       hybridoma technique (Kesber *et al.* 1983, *Immunology Today* 4:72) and EBV-hybridoma technique (Cole *et al.* 1985, *Monoclonal Antibodies* and

Cancer Therapy, Alan R. Liss, Inc. pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" by splicing the genes from an antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity may be used (Morrison *et al.* 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger *et al.* 1984 Nature 312:604-608; Takeda *et al.* 1985 Nature 31:452-454). Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce 4-specific single chain antibodies.

Recombinant human or humanized versions of monoclonal antibodies are a preferred embodiment for human therapeutic applications. Humanized antibodies may be prepared according to procedures in the literature (e.g. Jones *et al.* 1986, Nature 321:522-25; Reichman *et al.* 1988, Nature 332:323-27; Verhoeyen *et al.* 1988, Science 239:1534-36). The recently described "gene conversion mutagenesis" strategy for the production of humanized monoclonal antibody may also be employed in the production of humanized antibodies (Carter *et al.* 1992 Proc. Natl. Acad. Sci. U.S.A. 89:4285-89). Alternatively, techniques for generating the recombinant phage library of random combinations of heavy and light regions may be used to prepare recombinant antibodies (e.g. Huse *et al.* 1989 Science 246:1275-81).

Antibody fragments which contain the idiotype of the molecule such as  $F(ab^1)$  and  $F(ab^2)$  may be generated by known techniques. For example, such fragments include but are not limited to: the  $F(ab)$  E2 fragment which can be produced by pepsin digestion of the intact antibody molecule; the  $Fab'$  fragments which can be generated by reducing the disulfide bridges of the  $F(ab')_2$  fragment, and the two  $Fab$  fragments which can be generated by treating the antibody molecule with papain and a reducing agent. Alternatively,  $Fab$  expression libraries may be constructed (Huse *et al.* 1989, Science 246:1275-1281) to allow rapid and easy cloning of a monoclonal  $Fab$  fragment with the desired specificity to a protein.

### Adjuvants and Carriers

Pharmaceutically acceptable carriers or diluents include those used in compositions suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. They are

non-toxic to recipients at the dosages and concentrations employed. Representative examples of pharmaceutically acceptable carriers or diluents include, but are not limited to water, isotonic solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline) and can also contain one or more of, mannitol, lactose, trehalose, dextrose, glycerol, ethanol or polypeptides (such as human serum albumin). The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy.

As mentioned above the composition may include an adjuvant. As will be understood an "adjuvant" means a composition comprised of one or more substances that enhances the immunogenicity and efficacy of a vaccine composition. Non-limiting examples of suitable adjuvants include squalane and squalene (or other oils of animal origin); block copolymers; detergents such as Tween®-80; Quil® A, mineral oils such as Drakeol or Marcol, vegetable oils such as peanut oil; *Corynebacterium*-derived adjuvants such as *Corynebacterium parvum*; *Propionibacterium*-derived adjuvants such as *Propionibacterium acne*; *Mycobacterium bovis* (Bacille Calmette and Guerin or BCG); interleukins such as interleukin 2 and interleukin 12; monokines such as interleukin 1; tumour necrosis factor; interferons such as gamma interferon; combinations such as saponin-aluminium hydroxide or Quil-A aluminium hydroxide; liposomes; ISCOM adjuvant; mycobacterial cell wall extract; synthetic glycopeptides such as muramyl dipeptides or other derivatives; Avridine; Lipid A derivatives; dextran sulfate; DEAE-Dextran or with aluminium phosphate; carboxypolymethylene such as Carbopol' EMA; acrylic copolymer emulsions such as Neocryl A640 (e.g. U.S. Pat. No. 5,047,238); vaccinia or animal poxvirus proteins; sub-viral particle adjuvants such as cholera toxin, or mixtures thereof.

### Gene/DNA Isolation

The DNA encoding a protein may be obtained from any cDNA library prepared from tissue believed to express the gene mRNA and to express it at a detectable level. DNA can also be obtained from a genomic library.

Libraries are screened with probes or analytical tools designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes include monoclonal or polyclonal

antibodies that recognize and specifically bind the protein; oligonucleotides of about 20-80 bases in length that encode known or suspected portions of cDNA from the same or different species; and/or complementary or homologous cDNAs or fragments thereof that encode the same or a hybridizing gene. Appropriate probes for screening genomic DNA libraries include, but are not limited to, oligonucleotides; cDNAs or fragments thereof that encode the same or hybridizing DNA including expressed sequence tags and the like; and/or homologous genomic DNAs or fragments thereof. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in chapters 10-12 of Sambrook *et al.*

An alternative means to isolate a gene encoding the protein of interest is to use polymerase chain reaction (PCR) methodology as described in section 14 of Sambrook *et al.* This method requires the use of oligonucleotide probes that will hybridize to the gene.

The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The actual nucleotide sequence(s) is usually based on conserved or highly homologous nucleotide sequences or regions of the gene. The oligonucleotides may be degenerate at one or more positions. The use of degenerate oligonucleotides may be of particular importance where a library is screened from a species in which preferential codon usage in that species is known. The oligonucleotide must be labelled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labelling is to use ( $\alpha$ - $^{32}$ P)- dATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labelling.

DNA encompassing all the protein coding sequence is obtained by screening selected cDNA or genomic libraries, and if necessary, using conventional primer extension procedures as described in section 7.79 of Sambrook *et al.*, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Another alternative method for obtaining the gene of interest is to chemically synthesize it using one of the methods described in Fingels *et al.* (*Agnew Chem. Int. Ed. Engl.* 28: 716-734, 1989). These methods include

triesters, phosphite, phosphoramidite and H-Phosphonate methods, PCR and other autoprimer methods, and oligonucleotide syntheses on solid supports. These methods may be used if the entire nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding strand is available, or alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue.

#### **Substantially Purified**

By "substantially purified" we mean a polypeptide that has been separated from lipids, nucleic acids, other polypeptides, and other contaminating molecules.

#### **Hybridisation**

The polynucleotide sequence of the present invention may hybridise to the respective sequence set out SEQ. ID. NOS. 2, 4, or 6 under high stringency. As used herein, stringent conditions are those that (i) employ low ionic strength and high temperature for washing after hybridization, for example, 0.1 x SSC and 0.1% (w/v) SDS at 50°C; (ii) employ during hybridization conditions such that the hybridization temperature is 25°C lower than the duplex melting temperature of the hybridizing polynucleotides, for example 1.5 x SSPE, 10% (w/v) polyethylene glycol 6000, 7% (w/v) SDS, 0.25 mg/ml fragmented herring sperm DNA at 65°C; or (iii) for example, 0.5M sodium phosphate, pH 7.2, 5mM EDTA, 7% (w/v) SDS and 0.5% (w/v) BLOTTO at 70°C; or (iv) employ during hybridization a denaturing agent such as formamide, for example, 50% (v/v) formamide with 5 x SSC, 50mM sodium phosphate (pH 6.5) and 5 x Denhardt's solution (32) at 42°C; or (v) employ, for example, 50% (v/v) formamide, 5 x SSC, 50mM sodium phosphate (pH 6.8), 0.1% (w/v) sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50µg/ml) and 10% dextran sulphate at 42°C.

**EXAMPLE 1**

This example describes the cloning and characterisation of a protease from *Moraxella bovis*.

5

**Bacteria and construction of a genomic library**

*Moraxella bovis* strain Dalton 2d was a field isolate collected from a bovine eye and characterised by CSIRO Animal Health, Parkville, Australia (6). *Escherichia coli* strain DH5 $\alpha$  has been previously described (7, 8).

10 All enzymes were purchased from Promega (Madison, WI, USA) except where otherwise noted.

General cloning and DNA techniques were as described (9) unless otherwise noted.

15 A genomic library was constructed by carrying out partial *Sau*3A digests on genomic DNA extracted from *M. bovis* strain Dalton 2d using a CTAB method which is outlined below. This DNA was size fractionated using a NaCl gradient (10) and ligated with the cosmid cloning vector pHC79 (11) which had been previously digested with *Bam*HI. This DNA was packaged into lambda bacteriophage heads using the Packagene Lambda  
20 DNA packaging system (Promega, Madison, WI, USA) and this was used to transduce the *E. coli* strain DH5 $\alpha$ . The library was stored in 96 well trays (50% glycerol / luria broth / ampicillin (50 $\mu$ g/ml)) at -70°C.

**CTAB genomic DNA extraction from *M. bovis***

25 A 5ml brain heart infusion (BHI) (Oxoid Ltd., Basingstoke, Hampshire, U.K.) broth was inoculated with a colony of Dalton 2d taken from a fresh overnight culture on horse blood agar and incubated with shaking at 37°C for 6 hours. This culture was used to inoculate 50ml of BHI broth which was grown with shaking at 37°C overnight. 40ml of the culture was transferred to  
30 an SS34 tube and the cells pelleted at 3000  $\times$  g for 10 minutes. Following resuspension of the pellet in 9.5ml of 25% sucrose in TE buffer (10mM Tris, 1mM EDTA (pH8)), 500 $\mu$ l of 10% SDS, 50 $\mu$ l of 20mg/ml proteinase K and 20 $\mu$ l of 10mg/ml RnaseA were added and this mixture incubated in an orbital shaker for 1 hour at 37°C. To this mixture, 1.8ml of 5M NaCl and 1.5ml of a  
35 CTAB (N-Cetyl-N,N,N-trimethyl-ammonium bromide) / NaCl solution was added and incubation continued for 20 minutes at 65°C. The DNA was

extracted using phenol/chloroform and precipitated with 0.6 volumes of isopropanol. The resulting DNA was washed in 70% ethanol, dried and resuspended in 2ml of TE buffer.

**5 Screening of genomic library for enzyme activity**

The genomic library was cultured on skim milk agar to screen for the presence of a clone displaying protease activity (double strength Columbia agar base (Oxoid Ltd., Basingstoke, Hampshire, U.K.) / 10% skim milk) for 24 hours at 37°C followed by refrigeration at 4°C for one to two days.

10 A single clone from the genomic library was detected as having activity against skim milk agar. DNA analysis confirmed that the clone contained a fragment of *M. bovis* Dalton 2d genomic DNA approximately 40 kilobases in size. The construct was designated pJF1.

**15 Nucleotide sequence of the *M. bovis* protease clone pJF1**

Plasmid and cosmid DNA for automated sequencing was extracted using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) and the Qiagen Plasmid Midi Kit (Qiagen Pty. Ltd., Clifton Hill, Vic, Australia), respectively.

20 The nucleotide sequence of the insert DNA was determined using the process of "primer walking" (12). This was achieved using synthetic oligonucleotides (Bresatec / Geneworks, Thebarton, SA, Australia) and the dye terminator cycle sequencing ready reaction (Perkin Elmer Corporation, Norwalk, CT, USA). The resulting sequence was analysed on an Applied  
25 Biosystems 373A DNA sequencer.

Automated sequencing revealed an open reading frame of 3345bp capable of encoding a protein of 1115 amino acids. The sequence is written in the 5' to 3' direction and is shown in Figure 1 together with the corresponding amino acid sequence which is predicted to encode a protein  
30 with a molecular weight of 120kDa. The amino acid sequence is shown in SEQ. ID. NO. 1 and the DNA sequence is shown in SEQ. ID. NO. 2.

The putative start codon for the mature protease protein was identified by the presence of a possible ribosome binding site upstream. This RBS was identified by its similarity to the consensus sequence for the *E. coli* RBS and  
35 that previously identified for the *M. bovis* pilin genes (AGGAG) (27)

Due to the secreted nature of the protease, it was assumed that it would contain in its N-terminal sequence a signal peptide which would be used in the secretion of the protein. This analysis was carried out using a prediction program (SignalP) available through the Expasy website  
 5 (<http://www.expasy.ch/tools/>), which allows for the identification of prokaryotic signal peptides and predicts possible cleavage sites. This analysis only identified a signal peptide using the start codon indicated in the accompanying protein/DNA sequence.

#### 10 Sequence comparisons

Comparisons of the deduced amino acid sequence with those in the database were carried out using the BlastX and BlastP programs (13) which are available at <http://www.ncbi.nlm.nih.gov>.

At the amino acid level, the protease cloned from Dalton 2d displayed  
 15 the following similarity and identity to the proteins listed.

Organism	Protein	Similarity	Identity
<i>Serratia marcescens</i>	ssp-h2 - serine protease autotransporter	39%	23%
<i>Serratia marcescens</i>	ssp-h1 - serine protease autotransporter	37%	22%
<i>Pseudomonas fluorescens</i>	serine protease homologue	34%	20%
<i>Pseudomonas tolaasii</i>	serine protease	35%	21%

More generally the 5' domain of the *M. bovis* protease displays homology to a family of subtilisins (serine proteases) while the 3' region  
 20 resembles a number of outer membrane proteins.

The *M. bovis* sequence was found to contain a highly proline rich region which distinguished it from all other proteins to which it was closely related.

#### 25 Protease type encoded by pJF1

In order to identify the type of protease activity encoded by pJF1, a range of specific protease inhibitors were examined for their effect on the expression of the *M. bovis* protease.



The method of Bourgeau *et al.*, (1992) (14) was used to determine inhibitor activity with the following modifications. 100µl of cell free supernatant from a fresh overnight broth culture was mixed with 650µl of 100mM Tris (pH 7.2) and a suitable volume of inhibitor PMSF (phenylmethylsulfonyl fluoride) 5mM; EDTA 5mM; leupeptin 100µg/ml; pepstatin 50µg/ml. Distilled water was used to make the volume up to 1ml. The mixture was incubated at 37°C for 30 minutes and 10mg of azocoll (Calbiochem, Alexandria, NSW, Australia) was then added. The suspensions were incubated at 37°C for 16 hours and the optical density read at 520nm.

In this way it was confirmed that the activity attributable to the protease encoded by pJF1 was that of a serine protease since PMSF (a serine protease inhibitor) reduced the protease activity of both Dalton 2d and pJF1 to zero.

#### Conservation of protease in *M. bovis*

Southern hybridisation using an internal fragment of the protease coding region as a probe was carried out to investigate whether the protease was present in strains representing the known *M. bovis* pili serotypes.

Genomic DNA extracted from the representative strains of *M. bovis* (15) was digested with *Xba*I and *Eco*RI and separated using agarose gel electrophoresis. The DNA was transferred to a Hybond N+ filter (Amersham, Little Chalfont, Buckinghamshire, U.K.) using the method described (9). The probe used in the southern hybridisation was a PCR amplified fragment which was internal to the protease coding region. This fragment was labelled with  $\alpha^{32}$ P-dATP using the Megaprime labelling system (Amersham, Little Chalfont, Buckinghamshire, U.K.) according to the manufacturers instructions. High stringency conditions were used (hybridisation temperature 68°C; 2 washes at room temperature in 2 × SSC / 0.1% SDS; 1 wash at 68°C in 0.1 × SSC / 0.1% SDS) and the resulting filters were exposed to autoradiographic film (Kodak, Rochester, New York, USA) for 5 to 24 hours before developing.

Results showed that the protease gene cloned in pJF1 is present in all strains of *M. bovis* examined.

## **EXAMPLE 2**

This example describes the cloning and characterisation of a lipase from *Moraxella bovis*.

5

### **Bacteria and construction of a plasmid library**

*Moraxella bovis* strain Dalton 2d was a field isolate collected from a bovine eye and characterised by CSIRO Animal Health, Parkville, Australia (6). *Escherichia coli* strain MC1061 has been previously described (16).

10 All enzymes were purchased from Promega (Madison, WI, USA) except where otherwise noted. General cloning and DNA techniques were as described (9) unless otherwise noted.

A plasmid library was constructed in the cloning vector pBR322 (17). This was done by partially digesting genomic DNA extracted from Dalton 2d (using the CTAB method described in Example 1) with *Sau*3A under conditions that maximised the amount of DNA in the range of 1 to 2kb. This DNA was ligated with pBR322 which had been previously digested with *Bam*HI. The ligated DNA was electroporated (2.5kV, 200Ω and 200μF, for a theoretical time constant of 4.7) into electrocompetent *E. coli* MC1061 cells.

20

### **Screening of plasmid library for lipase expression**

Following electroporation of the ligated DNA into MC1061 cells, recombinant clones displaying lipase activity were detected by culturing the library for 24 hours at 37°C on media containing Tween 80 [10ml Tween 80 (Sigma, St Louis, MO, USA), 5g NaCl, 3g agar No.1 (Oxoid Ltd., Basingstoke, Hampshire, U.K.), 10g peptone, 0.1g CaCl<sub>2</sub>.H<sub>2</sub>O / litre].

25 Twenty eight out of 24,000 clones screened were found to be displaying lipase activity. DNA analysis confirmed that all of these clones contained one 5.4kb fragment of DNA in common. One clone was chosen to continue work with and this was designated pMB1.

30 In some experiments (below), a photometric assay of extracellular lipase activity was performed with p-nitrophenylpalmitate as the substrate (18, 19). Strains of *E. coli* and/or *M. bovis* were grown at 37°C for the required time points. Cell free culture supernatant (100μl) was mixed with 2.4ml of enzyme buffer (19) to assay secreted lipase activity. After 30 minutes incubation at 37°C, the optical density at 410nm was determined.

35

One enzyme unit was defined as the amount of enzyme that releases 1 nmol of p-nitrophenyl from p-nitrophenylpalmitate  $\text{ml}^{-1} \text{min}^{-1}$ . Under the conditions described by Stuer *et al.*, (18), an optical density at 410nm of 0.041 is equivalent to 1 enzyme unit.

5

#### Nucleotide sequence of the *M. bovis* lipase clone pMB1

Plasmid pMB1 was subjected to automated DNA sequencing using the methodology described in Example 1.

10 This analysis revealed an open reading frame of 1851bp capable of encoding 617 amino acids. The sequence is written in the 5' to 3' direction and is shown in Figure 2 together with the corresponding amino acid sequence that is predicted to encode a protein with a molecular weight of 65.8kDa. The amino acid sequence is shown in SEQ. ID. NO. 3 and the DNA sequence is shown in SEQ. ID. NO. 4.

15 The techniques set out above in respect of the protease were used to identify the potential start codon for the lipase protein.

#### Sequence comparisons

20 Sequence comparisons were made using the methodology described in Example 1.

At the amino acid level, the lipase cloned from *M. bovis* Dalton 2d was shown to display the following similarity and identity to the proteins listed.

Organism	Protein	Similarity	Identity
<i>Xenorhabdus luminescens</i>	triacylglycerol lipase	36%	24%
<i>Pseudomonas putida</i>	hypothetical protein	36%	24%
<i>Salmonella typhimurium</i>	outer membrane esterase	35%	23%
<i>Pseudomonas aeruginosa</i>	lipase / esterase	36%	23%

25 The *M. bovis* lipase was identified as being a possible new member of the GDSL family (20) of lipolytic enzymes.

#### N-terminal sequencing carried out on the lipase mature protein

The required strains of *E. coli* were cultured overnight with shaking at 37°C in 500mls of luria broth. The cells were pelleted at 5,000 rpm for 15 mins and the supernatant filtered through a 0.45µm filter. Solid ammonium sulfate was added to the supernatant to 60% saturation (180g / 500ml), and dissolved at 4°C with stirring for 30 minutes. This mixture was left at 4°C overnight and the precipitated proteins pelleted at 7,000 rpm for 30 mins. The proteins were resuspended in 3ml of double distilled water and the solubilised proteins dialysed against double distilled water overnight to remove any salt. The resulting mixture was filtered through a 0.45µm filter and stored at -20°C.

Following separation of the proteins by SDS-PAGE, the proteins were transferred to PVDF membrane and excised. The protein was subjected to automated (Edman degradation) sequence analysis (28) with vapour phase delivery of critical reagents (29) in an automated sequenator (model 470A; Applied Biosystems) (Applied Biosystems Division, Foster City, CA, USA) in conjunction with a PTH amino acid separation system (model 120A PTH analyzer; Applied Biosystems).

Using this technique 17 amino acids with two gaps were identified  
K E F S Q V I I F G D S L X D X G (SEQ ID NO:7)  
which corresponds exactly with amino acids 26 through to 42 shown on the accompanying sequence. This result also indicated that the protein most likely includes an amino terminal signal peptide which is involved in the secretion of the protein. This amino terminal corresponds to amino acids 1 through to 25 in the accompanying sequence.

#### **Raising antibodies to the lipase in rabbits**

Antibody to the recombinant lipase was raised in rabbits by injecting ammonium sulfate precipitated supernatant from *E. coli* MC1061/pMB4. Prior to vaccination, the lipase preparation was inactivated by heating to 90°C for 90min. 30µg of this protein was injected at 2 weekly intervals for 4 weeks. The primary inoculum was emulsified with Freund's complete adjuvant and subsequent vaccinations with Freund's incomplete adjuvant.

#### **Heat stability of *M. bovis* lipase**

The recombinant lipase cloned from *M. bovis* Dalton 2d was found to be very heat stable since it required heating at 90°C for 105 minutes for the

activity to be reduced by 97%. Figure 3 illustrates this phenomenon with enzyme activity expressed as "lipase enzyme units" as determined in the extracellular lipase assay.

#### 5    **Relative expression levels of native versus recombinant lipase**

          An experiment was performed to plot growth rate with lipase production and to compare production of the recombinant lipase from MC1061/pMB1 with that of the native form of the lipase from *M. bovis* Dalton 2d. Figure 4 illustrates that the two strains grow at approximately the same  
10    rate but they do not reach the same cell density, with Dalton 2d substantially lower after 9 hours than MC1061/pMB1. Lipase expression levels were greatest from the pMB1 construct in *E. coli* compared to native lipase expression from *M. bovis* Dalton 2d.

          This result was further substantiated when proteins from cell-free  
15    supernatants of either the *E. coli* clone or *M. bovis* Dalton 2d were ammonium sulfate precipitated and analysed by SDS-PAGE and western blot using antisera to the recombinant heat-deactivated lipase.

          Ammonium sulfate precipitated supernatants were prepared from overnight cultures of *E. coli* or *M. bovis* that had been shaken at 37°C in  
20    either 500mls of Luria broth or brain heart infusion broth, respectively. Cells were pelleted at 5000 × g for 15 minutes and the supernatant filtered through a 0.45µm filter. Solid ammonium sulfate was added to the supernatant to 60% saturation (180g / 500ml) and dissolved at 4°C with stirring for 30 minutes. This mixture was left at 4°C overnight and the precipitated protein  
25    pelleted at 7000 × g for 30 minutes. Proteins were resuspended in 3ml of double distilled water and the solubilised proteins dialysed against double distilled water overnight to remove any salt. The resulting mixture was filtered through a 0.45µm filter and stored at -20°C.

          Protein samples (100µl) were prepared for SDS-PAGE by resuspension  
30    in 100µl of 2x sample buffer (5ml 0.5M Tris (pH6.8), 8ml 10% SDS, 4ml glycerol, 0.8ml β-mercaptoethanol, 1ml double distilled H<sub>2</sub>O, bromophenol blue) and heating to 100°C for 5 minutes. The proteins were separated on a 12.5% polyacrylamide gel using the buffer system of Laemmli (21).

          Western blots were carried out according to the method of Towbin *et al.*, (22) and following separation of proteins by SDS-PAGE and transfer to  
35    nitrocellulose using the Bio-Rad minicell (Bio-Rad, Hercules, CA, USA)

transfer system. Filters were immunoblotted with the recombinant lipase antiserum (at a concentration of 1/100) which had been adsorbed against MC1061 cells. The antiserum was raised against ammonium sulfate precipitated recombinant lipase which had been heat deactivated (1 hour 45 minutes at 90°C) and used to inoculate rabbits (three doses of 50µg each) at 4 week intervals. Blood samples were collected from the marginal ear vein prior to immunisation and at each vaccination time point.

The results showed a prominent band present in the recombinant lipase positive construct MC1061/pMB1 that is detectable in relatively minor amounts in *M. bovis* Dalton 2d preparation. The protein detected with the antisera was approximately the same size as that of the predicted molecular weight for the *M. bovis* lipase (65.8kDa).

#### Lipase type encoded by pMB1

Thin layer chromatography (TLC) was used to determine whether the lipase of *M. bovis* Dalton 2d displayed phospholipase activity. Characterisation of phospholipase type essentially followed a previously described method (23) except that the results of separation on Silica Gel 60 plates were visualised by developing with a 10% ethanolic solution of molybdophosphoric acid at 100°C. All reagents used were purchased from Sigma (Sigma, St Louis, MO, USA).

TLC determined that the *M. bovis* lipase displayed the same enzyme specificity as that of a commercially-available phospholipase B when lysophosphatidylcholine and phosphatidylcholine were used as substrates (data not shown).

#### Conservation of lipase among *M. bovis*

A southern blot using an internal fragment of the Dalton 2d lipase coding region was used to investigate whether the lipase gene was present in strains of *M. bovis* representing the known pilus serotypes.

Genomic DNA extracted from the strains of *M. bovis* representing each of the known pilus serotypes (15) was digested with *HindIII* and separated using agarose gel electrophoresis. The DNA was transferred to a Hybond N+ filter (Amersham, Little Chalfont, Buckinghamshire, U.K.) using a previously described method (9). The probe used in the southern hybridisation was a *HindIII* fragment that contained sequence internal to the lipase coding

region. This fragment was labelled with  $\alpha^{32}\text{P}$ -dATP using the Megaprime  
labelling system (Amersham, Little Chalfont, Buckinghamshire, U.K.)  
according to the manufacturers instructions. High stringency conditions  
were used (hybridisation temperature 68°C; 2 washes at room temperature in  
5 2 × SSC / 0.1% SDS; 1 wash at 68°C in 0.1 × SSC / 0.1% SDS) and the  
resulting filters were exposed to autoradiographic film (Kodak, Rochester,  
New York, USA) for 5 to 24 hours before developing.

Results showed that the lipase gene is present in all strains of *M. bovis*  
examined.

10 To confirm whether or not the lipase gene was expressed in each of the  
serotype representative strains, antisera raised against recombinant heat  
deactivated lipase was used in a western blot analysis of whole cell  
preparations. Results showed that the lipase was indeed being expressed by  
all of these *M. bovis* strains.

15

### EXAMPLE 3

#### Bacteria and construction of a haemolysin clone

*Moraxella bovis* strain Dalton 2d was a field isolate collected from a  
20 bovine eye and characterised by CSIRO Animal Health, Parkville, Australia  
(6).

All of the *M. bovis* strains representative of the known pilus serotypes  
express a haemolytic activity that is detected on horse blood agar.

*Escherichia coli* strain degP4::Tn5 has a leaky outer membrane and is  
25 defective in proteolysis and has been previously described (24).

All enzymes were purchased from Promega (Madison, WI, USA) except  
where otherwise noted.

General cloning and DNA techniques were as described (9) unless  
otherwise noted.

30 A *phoA* fusion technique that allows for the identification of exported  
proteins (25) was utilised with some modifications. Genomic DNA from *M.*  
*bovis* Dalton 2d (prepared using the CTAB method described in Example 1)  
was partially digested with *Sau*3A. Restricted DNA was ligated with a series  
of vectors that allow fusions with an alkaline phosphatase gene in three  
35 different reading frames. The ligated DNA was electroporated into *E. coli*  
degP4::Tn5 and the resulting clones screened on Luria agar containing

ampicillin (50µg/ml) and X-P (200µg/ml) (5-bromo-3-chloro-indolyl phosphate). Selection of clones relies on the observation that if the fragment is cloned in frame and contains an export sequence the resulting colony will be blue in colour. The leaky *E. coli* strain allows the outer membrane-bound proteins and secreted proteins (both fused with *phoA*) to be distinguished from non-secreted fusion proteins.

#### Sequencing of the *M. bovis* haemolysin determinant

Clones selected for the presence of a secreted or outer membrane protein gene sequence were subjected to automated DNA sequencing using the methods described in Example 1. One of these clones, pMbh1, was found to contain 200bp of DNA which displayed high homology to the A subunit of other RTX toxins. Inverse PCR and degenerate oligonucleotides were utilised to obtain the sequence of the entire A subunit. The open reading frame of 2784bp was capable of encoding 928 amino acids. The sequence is written in the 5' to 3' direction and is shown in Figure 5 together with the corresponding amino acid sequence that is predicted to encode a protein with a molecular weight of 98.8kDa. The amino acid sequence is shown in SEQ. ID. NO. 5 and the DNA sequence is shown in SEQ. ID. NO. 6.

The putative start codon was identified using the RBS technique outlined above. A signal peptide analysis was not carried out as the A subunit is not secreted on its own. However as the protein sequence of these proteins (RTX) is quite highly conserved, on amino acid homologies alone this start codon was the one of choice.

25

#### Sequence homology

At the amino acid level the *M. bovis* Dalton 2d haemolysin gene product shows striking similarity to the A subunit of the of several RTX and other haemolysins as shown in the following table.



Organism	Protein	Similarity	Identity
<i>Pasteurella haemolytica</i>	LktA protein (leukotoxin)	68%	50%
<i>Actinobacillus pleuropneumoniae</i>	RTX toxin determinant	68%	48%
<i>Escherichia coli</i>	Haemolysin - plasmid	58%	43%
<i>E. coli</i>	Haemolysin - chromosomal	58%	43%

#### Functional complementation by the *M. bovis* haemolysin

A construct which expressed the chromosomal-borne haemolysin of *E. coli* was obtained (pLG900; generated by combining the two plasmids pLG575 (26) and pLG816 (*hlyC* and *hlyA* cloned into pBluescriptSK). pLG900 comprises the four genes of the RTX operon, *hlyC*, *hlyA*, *hlyB*, *hlyD*, cloned into pBluescriptSK and is capable of conferring a haemolytic phenotype on *E. coli* cells that were previously non-haemolytic. The A subunit (*hlyA*) of this construct was mutated such that it was no longer able to be expressed but the other genes involved in the operon (*hlyB*, *hlyC* and *hlyD*) remained intact. The *E. coli* strain containing this construct (pLG900 / *hlyA* negative) was no longer haemolytic. However, the haemolytic phenotype was restored by providing *in trans* the cloned haemolysin subunit gene from *M. bovis* Dalton 2d. Thus it was confirmed that the cloned *M. bovis* haemolysin gene encoded a structural subunit that was most probably a member of the RTX family of haemolytic enzymes.

Further sequence analysis has established that, like other members of the family, the *M. bovis* RTX A subunit gene is flanked by DNA sequences capable of encoding the RTX B,C and D proteins.

#### Conservation of the RTX A subunit among *M. bovis*

To determine whether the gene for the RTX A subunit was present in *M. bovis* strains representing the known pilus serotypes, a southern hybridisation analysis was performed using the coding region of the RTX A subunit as a probe.

Genomic DNA extracted from the seven serotype strains of *M. bovis* (15) was digested with *EcoRV* and separated using agarose gel electrophoresis. The DNA was transferred to a Hybond N+ filter (Amersham,

Little Chalfont, Buckinghamshire, U.K.) using a previously described method (9). The probe used was a PCR amplified product that contained all of the coding region from the A subunit of the RTX haemolysin of *M. bovis*. This fragment was labelled with  $\alpha^{32}\text{P}$ -dATP using the Megaprime labelling system (Amersham, Little Chalfont, Buckinghamshire, U.K.) according to the manufacturers instructions. High stringency conditions were used (hybridisation temperature 68°C; 2 washes at room temperature in  $2 \times \text{SSC} / 0.1\% \text{SDS}$ ; 1 wash at 68°C in  $0.1 \times \text{SSC} / 0.1\% \text{SDS}$ ) and the resulting filters were exposed to autoradiographic film (Kodak, Rochester, New York, USA) for 5 to 24 hours before developing.

Results showed that the gene encoding the RTX A haemolysin subunit was conserved in all seven representative strains of *M. bovis* examined. Interestingly, each of these strains is known to display the haemolytic phenotype on horse blood agar. In contrast, the non-haemolytic *M. bovis* strain Gordon 26L3 did not hybridise to the RTX A gene probe possibly suggesting that *M. bovis* contains only a single structural gene responsible for the haemolytic phenotype detected on horse blood agar.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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**Claims:**

1. A polypeptide, the polypeptide having an amino acid sequence as set out in SEQ. ID. NO. 1 from amino acid 37 to 1114, or a sequence having at least 50% identity thereto, or a functional fragment thereof.
2. A polypeptide as claimed in claim 1, the polypeptide having a sequence of at least 70% identity with the sequence shown in SEQ. ID. NO:1 from amino acid 37 to 1114.
3. A polypeptide as claimed in claim 1, the polypeptide having a sequence of at least 80% identity with the sequence shown in SEQ. ID. NO:1 from amino acid 37 to 1114.
4. A polypeptide as claimed in claim 1, the polypeptide having a sequence of at least 90% identity with the sequence shown in SEQ. ID. NO:1 from amino acid 37 to 1114.
5. A polypeptide as claimed in any one of claims 1 to 4, the polypeptide having protease activity.
6. A nucleic acid molecule, the nucleic acid molecule comprising a sequence encoding a polypeptide as claimed in any one of claims 1 to 5.
7. A nucleic acid molecule comprising a sequence as set out in SEQ. ID. NO:2 or a sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.
8. A nucleic acid molecule as claimed in claim 7, the nucleic acid molecule comprising a sequence having least 70% identity with the sequence shown in SEQ. ID. NO:2.
9. A nucleic acid molecule as claimed in claim 7, the nucleic acid molecule comprising a sequence having least 80% identity with the sequence shown in SEQ. ID. NO:2.

10. A nucleic acid molecule as claimed in claim 7, the nucleic acid molecule comprising a sequence having least 90% identity with the sequence shown in SEQ. ID. NO:2.
- 5 11. A composition for use in raising an immune response in an animal, the composition comprising the polypeptide as claimed in any one of claims 1 to 5 or a nucleic acid sequence as claimed in claim 6 and optionally a carrier and/or adjuvant.
- 10 12. A polypeptide, the polypeptide having an amino acid sequence as set out in SEQ. ID. NO:3 from amino acid 26 to 616, or a sequence having at least 50% identity thereto, or a functional fragment thereof.
- 15 13. A polypeptide as claimed in claim 12, the polypeptide having a sequence of at least 70% identity with the sequence shown in SEQ. ID. NO:3 from amino acid 26 to 616.
14. A polypeptide as claimed in claim 12, the polypeptide having a sequence of at least 80% identity with the sequence shown in SEQ. ID. NO:3  
20 from amino acid 26 to 616.
15. A polypeptide as claimed in claim 12, the polypeptide having a sequence of at least 90% identity with the sequence shown in SEQ. ID. NO:3 from amino acid 26 to 616.
- 25 16. A polypeptide as claimed in any one of claims 12 to 15, the polypeptide having lipase activity.
17. A nucleic acid molecule, the nucleic acid molecule comprising a  
30 sequence encoding a polypeptide of any one of claims 12 to 16.
18. A nucleic acid molecule comprising a sequence as set out in SEQ. ID. NO:4 or a sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.

19. A nucleic acid molecule as claimed in claim 18, the nucleic acid molecule comprising a sequence having at least 70% identity with the sequence shown in SEQ. ID. NO:4.
- 5 20. A nucleic acid molecule as claimed in claim 18, the nucleic acid molecule comprising a sequence having at least 80% identity with the sequence shown in SEQ. ID. NO:4.
- 10 21. A nucleic acid molecule as claimed in claim 18, the nucleic acid molecule comprising a sequence having at least 90% identity with the sequence shown in SEQ. ID. NO:4.
- 15 22. A composition for use in raising an immune response in an animal, the composition comprising a polypeptide as claimed in any one of claims 12 to 16 or a nucleic acid sequence as claimed in claim 17 and optionally a carrier and/or adjuvant.
- 20 23. A polypeptide, the polypeptide having an amino acid sequence as set out in SEQ. ID. NO:5, or a sequence having at least 60% identity thereto, or a functional fragment thereof.
24. A polypeptide as claimed in claim 23, the polypeptide having a sequence of at least 70% identity with the sequence shown in SEQ. ID. NO:5.
- 25 25. A polypeptide as claimed in claim 23, the polypeptide having a sequence of at least 80% identity with the sequence shown in SEQ. ID. NO:5.
26. A polypeptide as claimed in claim 23, the polypeptide having a sequence of at least 90% identity with the sequence shown in SEQ. ID. NO:5.
- 30 27. A polypeptide as claimed in any one of claims 23 to 26, the polypeptide having haemolysin activity.
28. A nucleic acid molecule, the nucleic acid molecule comprising a sequence encoding a polypeptide of any one of claims 23 to 27.
- 35

29. A nucleic acid molecule comprising a sequence as set out in SEQ. ID. NO:6 or a sequence having at least 60% identity thereto, or a sequence which hybridises thereto under stringent conditions.
- 5 30. A nucleic acid molecule as claimed in claim 29, the nucleic acid molecule comprising a sequence having at least 70 % identity with the sequence shown in SEQ. ID. NO:6.
- 10 31. A nucleic acid molecule as claimed in claim 29, the nucleic acid molecule comprising a sequence having at least 80 % identity with the sequence shown in SEQ. ID. NO:6.
- 15 32. A nucleic acid molecule as claimed in claim 29, the nucleic acid molecule comprising a sequence having at least 90 % identity with the sequence shown in SEQ. ID. NO:6.
- 20 33. A composition for use in raising an immune response in an animal, the composition comprising a polypeptide of any one of claims 23 to 27 or a nucleic acid sequence of claim 28 and optionally a carrier and/or adjuvant.
- 25 34. A composition for use in raising an immune response in an animal directed against *Moraxella*, the composition comprising at least one polypeptide selected from the group consisting of a polypeptide as claimed in any one of claims 1 to 5, a polypeptide as claimed in any one of claims 12 to 16, and a polypeptide as claimed in any one of claims 23 to 27, and optionally including an adjuvant or carrier.
- 30 35. A composition as claimed in claim 34, the composition comprising a polypeptide as claimed in any one of claims 23 to 27 and either one of, or preferably both of, a polypeptide as claimed in any one of claims 1 to 5 and a polypeptide as claimed in any one of claims 12 to 16.
- 35 36. A composition as claimed in claim 34 or claim 35 wherein the *Moraxella* is *M. bovis* or *M. catarrhalis*.



37. A composition as claimed in claim 34 or claim 35 wherein the *Moraxella* is *M. bovis*.
38. An antibody raised against a polypeptide selected from the group
- 5 consisting of a polypeptide as claimed in any one of claims 1 to 5, a polypeptide as claimed in any one of claims 12 to 16, and a polypeptide as claimed in any one of claims 23 to 27.

1/7

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1   ttctcatggt tgacagetta tcatcgataa gctttaatgc ggtagtttat cacagttaaa ttgctaaccg agtcaggcac cgtgtatgaa
91  atctaacaat gcgctcatcg tcatctctcg caccgtcacc ctggatgctg taggcatagg ctgggttatg ccggtactgc cgggectctt
181 gcgggatate gtccattccg acagcatcgc cagtcactat ggcgtgctgc tagcgctata tgcgttgatg caatttctat gcgcacccgt
271 tctcggagca ctgtccgacc gctttggcgg ccgcccagtc ctgctcgctt cgtactttgg agccactatc gactacgcga tcatggcgac
361 cacaccgcgc ctgtggatca ataattaatg aacatatata ctctatttaa tatttcttat ttattcgtaa tattgccata aaaataaac
451 attatttcta tattaactaa actgttaata ttgttaata ataaacattt gtttatctaa aaaaataaat aatataaac aagcaattac
541 aatcttattt ttgaaaatac aataatactg caattgctta atctagacat taagtttatt ttgtattaaa attgccaaaa ctgtgtgtaa
631 taagtttcac cgaattgata ctttaagggt atcaattatg caacatggta aatgatttct atgtttgttg gcatggcaca aatgtcttat
721 aataacttgt tatggatgat tgatggcaat gataaactta gtgacaatga taaacgcaa gaaggtgtaat atgtcattac aaactcaacc
1   M S L Q T Q
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7   P A K R G F Y V K P L S M A C H L V I S A S S T V S Y A N S
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127  Q A N L K R N Q P A P S A G T R T G Y S V M D T S N N S N L
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1351 tgtgattgat acaggcatta accgcttcaa ccgagacttg gttggtgcaa atgtgcatga tacacagatt gagtggtttt ctgctggcag
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307  A Q R L N Y N P T T G Q I N P N P Y R T S I T N A E V T L P
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```

Figure 1

2/7

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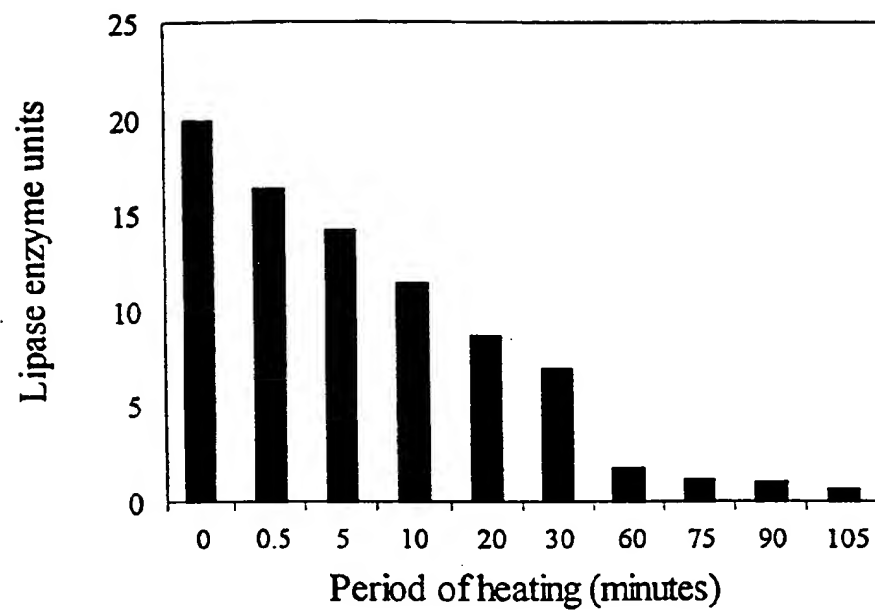
Figure 1 (continued)

3/7

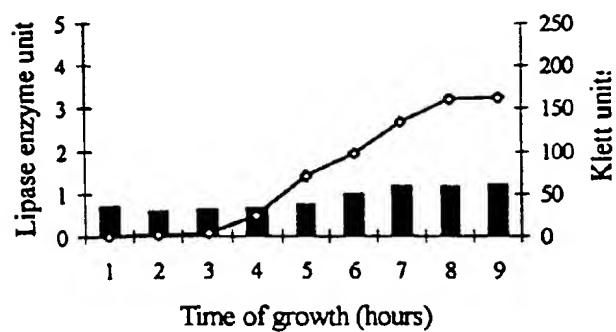
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 2071 atgataagct gtcaaacatg ag

Figure 2

4/7

**Figure 3**

5/7

(i) Dalton 2d / *M. bovis*

## (ii) pMB1 / MC1061

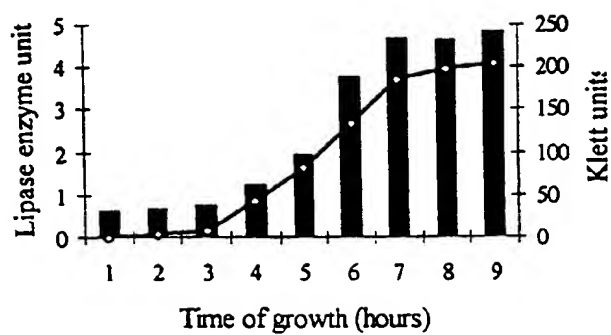


Figure 4

6/7

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Figure 5

7/7

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914 P I T Q P T Q G I L A P S V -

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Figure 5 (continued)



1/14

## SEQUENCE LISTING

<110> The University of Melbourne  
Commonwealth Scientific and Industrial Research Organisation

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Tyr Ala Asn Ser Ala Pro Met Ile Val Asp Ser Gln Tyr Asn Ser Ser
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Lys Tyr Ser Phe Tyr Asp Tyr Tyr Leu Asp Phe Leu Lys Arg Phe Arg
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Pro Thr Pro Thr Pro Val Pro Ser Pro Val Arg Pro Ala Pro Glu Leu
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Val Arg Pro Thr Pro Ala Pro Ile Pro Ala Pro Thr Pro Val Pro Thr
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Pro Ala Pro Ile Ser Gly Gly Ile Ser Gly Ser Tyr Ile Ala Pro Val
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Asn Leu Lys Arg Asn Gln Pro Ala Pro Ser Ala Gly Thr Arg Thr Gly
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Ile Asn Arg Phe Asn Arg Asp Leu Val Gly Ala Asn Val His Asp Thr
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2/14

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 Lys Ile Phe Asn Asn Ser Trp Gly Ser Asn Asn Thr Asp Gln Trp Tyr  
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3/14

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4/14

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5/14

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6/14

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Lys Thr Ala Ser Ala Asn Thr Pro Tyr Asn Pro Thr Gly Thr Asn Tyr
          85             90             95

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Ala Val Gly Gly Ala Arg Ser Gly Ser Glu Val Asn Trp Asn Gly Phe
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7/14

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Asp Leu Ser Leu Thr Pro Arg Ala Ile Tyr Gly Glu Ser Leu Met Ala  
 195 200 205

Gly Val Gln Asp Lys Ala Lys Leu Ala Ser Ser Leu Tyr Asn Ser Gly  
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Phe Lys Asn Thr Gln Gly Val Ala Cys Gln Met Pro Ala Arg Thr Thr  
 260 265 270

Gly Ala Asp Asp Val Ala Ser Thr Ser Leu Ala Cys Thr Lys Ala Asn  
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Leu Ile Glu Asn Gly Ala Asn Asp Thr Tyr Ala Phe Ala Asp Asp Ile  
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His Pro Ser Gly Arg Thr His Arg Ile Leu Ala Gln Tyr Tyr Arg Ser  
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Arg Thr Asp Pro Thr Thr Gln Ile Gly Leu Asp Val Ala Gly Ser Ser  
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His Arg His Asp Ile Gly Asn Val Arg Leu Lys Gly Val Ala Gly Ile  
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8/14

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9/14

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10/14

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11/14

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12/14

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13/14

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14/14

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU00/01048

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int. Cl. <sup>7</sup> : C07K 14/22, 16/12; A61K 38/48, 39/095; C12N 15/31		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) ANGIS: SEQ ID Nos. 1, 3, 5 ESPACE: keywords: Moraxella, antigen		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 90/07525, A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA (12 July 1990) see whole document	1-38
A	EP 0 146 523, A2 (NORDEN LABORATORIES) (26 June 1985) see whole document	1-38
A	Journal of Clinical Microbiology, vol. 13, No. 2, (February 1981), pages 269-271, Sandra K. Frank <i>et al.</i> , "Hydrolytic Enzymes of <i>Moraxella bovis</i> ". see whole document	1-38
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 16 October 2000		Date of mailing of the international search report 23 OCT 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer  DAVID GRIFFITHS Telephone No. (02) 6283 2628

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/AU00/01048

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FEMS Microbiology Letters, Vol. 124 (1994) pages 69-73, F. Mark Billson <i>et al.</i> , "A haemolytic cell-free preparation of <i>Moraxella bovis</i> confers protection against Infectious Bovine Keratoconjunctivitis". see whole document	1-38
A	American Journal of Veterinary Research, Vol. 51, No. 2 February 1990, pages 191-196, Patricia S. Høien-Dalen <i>et al.</i> , "Comparative characterization of the leukocidal and hemolytic activity of <i>Moraxella bovis</i> ". see whole document	1-38



**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.  
**PCT/AU00/01048**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
EP	146523	DE	3482442	DK	5950/84	ES	538474
		ES	8608320	GR	81194	IE	57878
		JP	60-146831	NZ	210490	PT	79640
		US	4675176	ZA	8409616		
WO	90/07525	AU	50886/90				
							END OF ANNEX

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